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(54) Retinoic acid receptor composition.

(57) A novel retinoic acid receptor is disclosed. The novel receptor is encoded for by cDNA carried on plasmid phRAR1, which has been deposited with the American Type Culture Collection for patent purposes. Chimeric receptor proteins are also disclosed. The chimera are constructed by exchanging functional domains between the glucocorticoid, the mineralocorticoid, the estrogen-related, the thyroid and the retinoic acid receptors. In addition, a novel method for identifying functional ligands for receptor proteins is disclosed. The method, which takes advantage of the modular structure of the hormone receptors and the idea that the functional domains may be interchangeable, replaces the DNA-binding domain of a putative novel receptor with the DNA-binding domain of a known receptor such as the glucocorticoid receptor. The resulting chimeric construction, when expressed in cells, produces a hybrid receptor whose activation of a ligand-(*e.g.*, glucocorticoid) inducible promoter is dependent on the presence of the new ligand. The novel method is illustrated in part by showing that the ligand for the new receptor protein is the retinoid, retinoic acid.

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FIELD OF THE INVENTION

The present invention relates generally to ligand-responsive regulatory proteins and genes encoding them. More particularly, the present invention relates to retinoid related regulatory proteins and genes encoding them, modification of these and other regulatory proteins and genes by recombinant DNA and other genetic engineering techniques, plus uses of the retinoid related regulatory proteins and genes, both unmodified and modified.

In addition the invention relates to a novel method for identifying functional ligands for ligand-responsive proteins. This method is especially useful for identifying functional ligand(s) for newly discovered receptor proteins. The method is exemplified in part by showing that a vitamin A related morphogen, retinoic acid, is a functional ligand for a newly discovered retinoid receptor protein.

BACKGROUND OF THE INVENTION

A central problem in eukaryotic molecular biology continues to be elucidation of molecules and mechanisms that mediate specific gene regulation in response to exogenous inducers such as hormones or growth factors. Although much remains to be learned about the specifics of such mechanisms, it is known that exogenous inducers such as hormones modulate gene transcription by acting in concert with intracellular components, including intracellular receptors and discrete DNA known as hormone response elements or HRE's.

More specifically, it is known that hormones like the glucocorticoid and thyroid hormones enter cells by facilitated diffusion. It is also known that the hormones then bind to specific receptor proteins, thereby creating a hormone/receptor complex. The binding of hormone to the receptor is believed to initiate an alosteric alteration of the receptor protein. As a result of this alteration, it is believed that the hormone/receptor complex is capable of binding with high affinity to certain specific sites on the chromatin DNA. Such sites, which are referred to in the art by a variety of names, including hormone response elements or HRE's, modulate expression (transcription of RNA) of nearby target gene promoters.

A major obstacle to further understanding the specifics of gene regulation by exogenous inducers such as hormones has been the lack of availability of receptor proteins in sufficient quantity and purity to allow such proteins to be adequately analyzed and characterized. This same lack of availability has thwarted the use of receptors in diagnostic assays to determine the presence of exogenous inducers (*e.g.*, the hormones) in various body fluids and tissues, as well as their use as "prototypes" for engineering chimeric receptor protein analogs.

In an effort to overcome this lack of availability of receptor proteins, co-pending application U.S.S.N. 108,471, which has been assigned to the Salk Institute for Biological Studies, assignee of the present application, discloses cloned genes for a variety of receptor proteins, including glucocorticoid-, thyroid-, mineralocorticoid- and new steroid-related receptors. U.S.S.N 108,471 further discloses detailed bio-chemical characterization of these molecules which shows that the receptor proteins contain discrete DNA- and ligand-binding domains. (Portions of U.S.S.N. 108,471 have been published; for portions relating to cloning of the glucocorticoid receptor and characterization of this molecule into discrete domains, see Hollenberg, *et al.* (1985) and Giguere, *et al.*, (1986); for other related work regarding receptors; *see* Hollenberg, *et al.*, (1987), Green, *et al.*, (1986), Green and Chambon, (1987), Kumar, *et al.*, (1987), Miesfeld, *et al.*, (1987) and Evans (1988)). USSN 108,471 corresponds to WO88-03168.

Further with regard to biochemical characterization of the receptors, sequence analysis of the human glucocorticoid receptor gene revealed homology with the product of the v-*erb*-*A* oncogene of avian erythroblastosis virus (AEV) (*see* Weinberger, *et al.*, (1985)). This group and others subsequently demonstrated the cellular homolog of v-*erb*-A to be the *beta* thyroid hormone receptor (*see* Weinberger *et al.*, (1986) and Sap, *et al.*, (1986)).

The discovery that the DNA-binding domain of the steroid and thyroid hormone receptors is highly conserved raised the question of whether this segment might be diagnostic for related ligand inducible transcription factors. It also raised the question of whether the DNA sequences encoding these domains might be used as hybridization probes to scan the genome for related, but novel, ligand-responsive receptors. Utilizing this approach, our group at the Salk Institute have identified several new gene products. As is shown in U.S.S.N. 108,471, one is the human aldosterone receptor (hMR, ATCC No. 67201) (*see* Arriza, *et al.*, (1987) for the published version of this portion of U.S.S.N. 108,471); a second is a novel thyroid hormone receptor expressed at high levels in the rat central nervous system (rTR *alpha*, ATCC No. 67281) (*see* Thompson, *et al.*, (1987) for th published version of this portion of U.S.S.N. 108,471).

referred to as hRARβ encodes human retinoic acid receptor *beta*. *See* Brand *et al.*, (1988).

As used herein, GR means glucocorticoid receptor. The DNA referred to as hGR codes for human glucocorticoid receptor GR. hGR is encoded by deposited pRShGR which has been accorded ATCC No. 67200.

As used herein, MR means mineralocorticoid receptor. The DNA referred to as hMR codes for human mineralocorticoid receptor MR. hMR is encoded by deposited pRShMR which has been accorded ATCC No. 67201.

As used herein, TR means thyroid receptor. TR*alpha* and TR*beta* refer to the *alpha* and *beta* forms of the thyroid receptor. The DNA's referred to as c-*erb*-*A*, h*erb*-*A* 8.7, peA101, rbeA12, and hFA8 all code for thyroid receptors. Plasmid ph*erb*-A 8.7 encodes hTRα; it has been deposited for patent purposes and accorded ATCC No. 40374. Plasmid peA101 encodes hTRβ; it has been deposited for patent purposes and accorded ATCC No. 67244. Plasmid rbeA12 encodes rTRα; it has been deposited for patent purposes and accorded ATCC No. 67281. Plasmid phFA8 encodes a partial clone of hTRα that has a deletion in the "ligand-binding" region of the clone (*i.e.*, the DNA that codes for the carboxy terminal end of the receptor protein). Plasmid phFA8 has been accorded ATCC No. 40372.

As used herein, ERR means estrogen-related receptor. The acronyms, hERR1 and hERR2 refer to human estrogen-related receptors 1 and 2. These receptors are more related to steroid receptors than to the thyroid receptors, yet they do not bind any of the major classes of known steroid hormones (Giguere, *et al*, 1988). hERR1 is encoded by deposited plasmids pE4 and pHKA, which have been accorded ATCC No. 67309 and 67310, respectively. (Neither pE4 or pHKA are complete clones; hERR1 is constructed by joining segments from both clones.) hERR2 is encoded by deposited plasmid phH3 which has been accorded ATCC No. 40373.

As used herein, VDR means vitamin D_3 receptor.

As used herein, MTV means mammary tumor virus; MMTV means mouse mammary tumor virus.

As used herein, RSV means Rous sarcoma virus; SV means Simian virus.

As used herein, *CAT* means chloramphenicol acetyltransferase.

As used herein, luciferase means firefly luciferase. See, de Wet, I.R., Wood, K.V., DeLuca, M., Helinski, D.R., and Subramani, S., Mol.Cell.Biol. 7: 725-737 (1987).

As used herein, COS means monkey kidney cells which express T antigen (Tag). *See* Gluzman, *Cell*, 23:175 (1981). COS cells are receptor-deficient cells that are useful in the functional ligand identification assay of the present invention.

As used herein, CV-1 means mouse kidney cells from the cell line referred to as "CV-1". CV-1 is the parental line of COS. Unlike COS cells, which have been transformed to express SV40 T antigen (Tag), CV-1 cells do not express T antigen. CV-1 cells are receptor-deficient cells that are also useful in the functional ligand identification assay of the present invention.

As used herein, the generic terms of art, "hormone response elements" or "HRE's", "transcriptional control units", "hormone responsive promoter/enhancer elements", "enhancer-like DNA sequences" and "DNA sequences which mediate transcriptional stimulation", all mean the same thing, namely, short *cis*-acting sequences (about 20 bp in size) that are required for hormonal (or ligand) activation of transcription. The attachment of these elements to an otherwise hormone-nonresponsive gene causes that gene to become hormone responsive. These sequences, referred to most frequently as hormone response elements or HRE's, function in a position- and orientation-independent fashion. Unlike other enhancers, the activity of the HRE's is dependent upon the presence or absence of ligand. (*See* Evans (1988) and the references cited therein.) In the present specification and claims, the phrase "hormone response element" is used in a generic sense to mean and embody the functional characteristics implied by all terms used in the art to describe these sequences.

As used herein, synthetic HRE's refer to HRE's that have been synthesized *in vitro* using automated nucleotide synthesis machines. Since the HRE's are only about 20 bp in size, they are easily synthesized in this manner. If wild-type, engineered or synthetic HREs are linked to hormone-nonresponsive promoters, these promoters become hormone responsive. *See* Evans (1988) and the references cited therein.

As used herein, the acronym GRE means glucocorticoid response element and TRE means thyroid receptor response element. GRE's are hormone response elements that confer glucocorticoid responsiveness via interaction with the GR. *See* Payvar, *et al.*, Cell, 35:381 (1983) and Schiedereit, *et al.*, Nature, 304:749 (1983). GRE's can be used with any wild-type or chimeric receptor whose DNA-binding domain can functionally bind (*i.e.*, activate) with the GRE. For example, sinc GR, MR and PR receptors can all activate GRE's, a GRE can be used with any wild-type or chimeric receptor that has a GR, MR or PR-type DNA-binding domain. TRE's are similar to GRE's except that they confer thyroid hormone responsiveness via interaction with TR. TRE's can be used with any wild-type or chimeric receptor whose DNA-

the first common site can be introduced immediately preceding the DNA-binding domain, the second common site immediately following it. (For example, in any of the steroid hormone superfamily of receptors that are shown in Figure 8, a unique *NotI* site can be introduced immediately preceding the DNA-binding domain and a unique *XhoI* site can be introduced immediately following it. This divides the receptors into three functional regions or "cassettes"; (1) an N-terminus cassette, (2) a DNA-binding domain cassette, and (3) a ligand-binding domain cassette. The three regions or cassettes from any one receptor can be combined with cassettes from other receptors to create a variety of chimeric receptors.

As used herein, the nomenclature used to identify the chimeric receptors is as follows: The various functional domains (N-terminus, DNA-binding and ligand-binding) are identified according to the "parental" receptor from which they originated. For example, domains from GR are "G" domains; TR domains are "T" domains (unless otherwise further specified as being "T_a" or "T_b" domains); MR domains are "M" domains; RAR domains are "R" domains (unless otherwise further specified as being "R_a" or "R_b" domains), and ERR domains are "E" domains (unless otherwise specified as being "E_1" or "E_2" domains). According to this notation, unless otherwise specified, "T" is used generically to mean either the T_3R_α or the T_3R_β receptors; "E" means either hERR1 or hERR2; and "R" means either the RARα or the RARβ receptors. Wild-type receptors do not contain any exchanged domains, and so according to this notation system would be identified as G-G-G (or GGG), $T_a-T_a-T_a$ (or $T_aT_aT_a$), $T_b-T_b-T_b$ (or $T_bT_bT_b$), M-M-M (or MMM), $R_a-R_a-R_a$ (or $R_aR_aR_a$), $R_b-R_b-R_b$ (or $R_bR_bR_b$), $E_1-E_1-E_1$ or $E_2-E_2-E_2$, where the first domain listed is the N-terminus domain, the middle domain is the DNA-binding domain, and the last domain is the ligand-binding domain. Any chimeric receptor will have functional domains from at least two wild-type or parental sources. For example, the chimeric receptor GGR_a would have N-termimus and DNA-binding domains from glucocorticoid receptor and the ligand-binding domain from the alpha retinoic acid receptor;

$$GT_aR_b$$

would have the N-terminus from glucocorticoid, the DNA-binding domain from thyroid receptor *alpha* and the ligand-binding domain from retinoic acid receptor *beta*.

As used herein, hGR_{NX}, $hTR\beta_{NX}$, and hRR_{NX} refer to hGR, hTRβ and hRR receptors that have been engineered to contain the unique sites for *NotI* and *XhoI* flanking the boundaries for the DNA-binding domains in these receptors. These mutant receptors exemplify construction of hybrid receptors that are comprised of all possible combinations of amino termini, DNA-binding domains, and ligand-binding domains from hGR, hMR, hERR1, hERR2, hTRα, hTRβ, rTRα, hRARα, and hRARβ.

As used herein, Southern blot analysis refers to a procedure for transferring denatured DNA from an agarose gel to a nitrocellulose filter where it can be hybridized with a complementary nucleic acid.

As used herein, Northern blot analysis refers to a technique for transferring RNA from an agarose gel to a nitrocellulose filter on which it can be hybridized to complementary DNA.

As used herein, "mutant" DNA of the invention refers to DNA which has been genetically engineered to be different from the "wild-type" or unmodified sequence. Such genetic engineering can include the insertion of new nucleotides into wild-type sequences, deletion of nucleotides from wild-type sequences, substitution of nucleotides in the wild-type sequences, or "swapping" of functional domains from one receptor to another. Receptors that have been engineered by "swapping" functional domains from one receptor to another are also referred to as chimeric or hybrid receptors. Chimeric receptors can be further engineered to include new nucleotides, deletion of nucleotides, substitution of nucleotides, etc.

Use of the term "substantial sequence homology" in the present specification and claims means it is intended that DNA, RNA, or amino acid sequences which have slight and non-consequential sequence variations from the actual sequences disclosed and claimed herein are within the scope of the appended claims. In this regard, the "slight and non-consequential" sequence variations mean that the homologous sequences will function in substantially the same manner to produce substantially the same compositions as the nucleic acid and amino acid compositions disclosed and claimed herein.

As used herein, the term "recombinantly produced" means made using genetic engineering techniques, not merely purified from nature.

The amino acids which comprise the various amino acid sequences appearing herein may be identified according to the following three-letter or one-letter abbreviations:

pRShGR (hGR)	67200	Sept. 9, 1986
pRShMR (hMR)	67201	Sept. 9, 1986
pE4 (hERR1*)	67309	Jan. 30, 1987
phHKA (hERR1*)	67310	Jan. 30, 1987
phH3 (hERR2)	40373	Sept. 29, 1987
GMCAT (reporter)	67282	Dec. 18, 1986
pherb – A 8.7 (hTRa)	40374	Sept. 29, 1987
phFA 8 (hTRa*)	40372	Sept. 29, 1987
peA101 (hTRb)	67244	Oct. 22, 1986
prbeA12 (rTRa)	67281	Dec. 18, 1986
phRARa (hRARa)	40392	Nov. 20, 1987

(* means a partial clone)
(pE4 & phHKA together encode complete hERR1)

SUMMARY OF THE INVENTION

In one aspect, the present invention comprises a double – stranded DNA segment wherein the plus or sense strand of the segment contains a sequence of triplets coding for the primary sequence of a protein which has ligand – binding and DNA – binding (or transcription – activating) properties characteristic of a retinoid receptor protein referred to herein as human retinoic acid receptor protein. According to this aspect of the invention, the double – stranded DNA segment is one which is capable of being expressed into retinoic acid receptor protein.

In another aspect, the invention comprises a single – stranded DNA, which is the sense strand of a double – stranded DNA coding for retinoic acid receptor protein, and an RNA made by transcription of this double – stranded DNA.

In another aspect, the invention comprises a plasmid, phRAR1, which contains DNA coding for a retinoic acid receptor protein of the present invention (RARα). This plasmid has been deposited with the American Type Culture Collection for patent purposes; it has been accorded ATCC No. 40392.

In still another aspect, the invention comprises a cell, preferably a mammalian cell, transformed with a DNA coding for retinoic acid receptor protein. According to this aspect of the invention, the transforming DNA is capable of being expressed in the cell, thereby increasing the amount of retinoic acid receptor, encoded by this DNA, in the cell.

Further the invention comprises novel retinoic acid receptors made by expression of a DNA coding for retinoic acid receptor or translation of an mRNA transcribed from such a retinoic acid receptor coding DNA. According to this aspect of the invention, the retinoic acid receptors will be protein products of "unmodified" retinoic acid coding DNA's and mRNA's, or will be modified or genetically engineered retinoic acid receptor protein products which, as a result of engineered mutations in the receptor DNA sequences, will have one or more differences in amino acid sequence from the corresponding naturally occurring "wild – type" retinoic acid receptor proteins. Preferably these retinoic acid receptors, whether "unmodified" or "engineered", will have at least about 5% of the retinoic acid binding activity and/or at least about 5% of the DNA – binding or transcription – activating activity of the corresponding naturally occurring retinoic acid receptor.

Further the invention comprises chimeric receptors made by exchanging the functional domains of one receptor with functional domains of another type. The chimeric DNA's thus produced encode chimeric receptor proteins that have functional characteristics based on the "origin" of their respective DNA – and ligand – binding domains. The chimeric receptors of the invention include double – stranded DNA's that code for the chimeric receptors, as well as single – stranded DNA's which are the sense strands of the double – stranded DNA's, and mRNA's made by transcription of the double – stranded DNA's. The invention also comprises cells, both eukaryotic and prokaryotic, that are transformed with chimeric receptors encoding DNA's of the invention.

According to the chimeric receptor aspect of the invention, to effect the chimeric DNA fusions, two restriction endonuclease sites are introduced into each receptor cDNA at comparable locations in or near the DNA – binding domains in order to divide the receptor DNA's into three functional domains or regions. (For example, a unique *Not*I site can be introduced immediately preceding the DNA – binding domain and a unique *Xho*I site can be introduced immediately following it. This divides the receptors into three functional regions or "cassettes"; (1) an N – terminus cassette, (2) a DNA – binding domain cassette, and (3) a ligand –

DNA sequences, *vis-à-vis* the ligand(s) that induce expression of the reporter gene.

As those skilled in the art will appreciate, if a cell already contains (a) a chimeric DNA sequence (C) comprised of (1) operative portions of a DNA-binding domain of a first receptor sequence (*i.e.*, a first sequence) linked to (2) operative portions of a ligand-binding domain of a second receptor sequence (*i.e.*, a second sequence), and (b) a reporter nucleic acid sequence functionally linked to an operative hormone response element wherein the operative portions of the DNA-binding domain of the first receptor sequence can functionally bind to and activate the hormone response element that is functionally linked to the reporter sequence, then the method for identifying a functional ligand for a receptor protein will be comprised of challenging the cell with at least one candidate ligand and then monitoring induction of the reporter sequence by means of changes in the amount of expression product of the reporter sequence.

The new functional ligand identification assay makes it possible to screen a large number of potential ligands or any given receptors, regardless of whether the receptor is a wild-type receptor or a chimeric one.

The functional ligand identification method is illustrated herein by showing (1) that the retinoid, retinoic acid and its metabolic precurser, retinol, are functional ligands for the receptor protein coded for by phRAR1 DNA, and (2) that the DNA- and ligand-binding domains determine the functional characteristics of the chimeric receptors.

The new functional assay, as well as the new retinoic acid receptor and the new chimeric receptors, are described more fully below.

DESCRIPTION OF THE INVENTION

The Retinoic Acid Receptor

In a continuing effort to explore the steroid hormone receptor superfamily, advantage was taken of the fortuitous identification of a novel genomic sequence with striking homology to the DNA-binding domain of the steroid hormone receptors (*see* Dejean *et al.*, 1986). This sequence spans the integration site of a hepatitis B virus (HBV) from a human hepatocellular carcinoma.

To pursue the hypothesis that this gene might code for a previously unknown receptor, an oligonucleotide derived from this sequence was labeled and used to probe a number of human cDNA libraries. Five positive clones were initially isolated from a testis cDNA library. The insert from one of these clones (1hT1R) was used to isolate additional cDNA clones from a λgt10 kidney cDNA library. A restriction map of the largest clone (phRAR1) is shown in Figure 1A. Nucleotide sequence analysis reveals a long open reading frame of 462 amino acids beginning with a presumptive initiator methionine codon corresponding to nucleotides 103-105 as shown in Fig. 1B-1. The sequence surrounding this ATG agrees with the consensus described by Kozak (1987) for a translation initiation site. Upstream of the ATG is an in-frame terminator providing support for the initiator methionine. Another methionine found 30 codons downstream fails to conform to the consensus and is an unlikely initiator. Following the terminator codon at position 1489-1491 is a 3'-untranslated region with a consensus polyadenylation signal (AATAAA) found 20 nucleotides upstream of a polyadenylated tract (*see* Proudfoot, et *al.*, 1976).

A polypeptide of relative molecular mass 50,772 d (51 Kd) is encoded within the translational open reading frame. The size of the protein encoded by the insert of phRAR1 was verified by *in vitro* translation of RNA (*see* Krieg, *et al.*, (1984)) derived from this insert and found to correspond to the predicted size of 54 Kd (data not shown). Amino acid sequence of this protein has been compared to the glucocorticoid and thyroid hormone receptors. The highest degree of similarity is found in a cysteine-rich sequence of 66 amino acids beginning at residue 88. Our group has previously demonstrated that this region of the hGR represents the DNA-binding domain for this receptor. *See* Giguere, *et al.*, (1987) and Hollenberg, *et al.*, (1987). In addition, mutagenesis and expression studies have provided direct evidence for its role in transcriptional activation of genes harboring glucocorticoid response elements (GREs). *See* Giguere, *et al.*, (1987) and Hollenberg, *et al.*, (1987).

Domain Switching and Transcriptional Activation

Since the ligand for the gene product of phRAR1 was unknown, it was desirabl to develop a quick and sensitive assay to reveal its identity. Previous studies have demonstrated that the DNA-binding domain of the human glucocorticoid and estrogen receptors can be interchanged to yield a functional hybrid receptor. This chimera recognizes the glucocorticoid responsive element of the MMTV-LTR but stimulates transcription in an estrogen-dependent fashion (*see* Green, *et al.*, (1987)). This led us to wonder is if a general

from phRAR1 reveals a major RNA species of 3,200 nucleotides with highest levels in the hippocampus, adrenals, cerebellum, hypothalamus and testis (Fig. 5). Longer exposure shows that most tissues contain a small amount of the 3.2 kb transcript while it is undetectable in some tissues such as liver.

Retinoic Acid Receptor Data Summary

The data disclosed herein identify the gene product of phRAR1 as a human retinoic acid receptor based on three criteria. First, the overall structural homology of the hRR to steroid and thyroid hormone receptors (Fig. 6) suggests that it is likely to be a ligand-responsive regulatory protein. Second, an expressed chimeric receptor, consisting of the DNA-binding domain of the hGR and the presumptive ligand-binding domain of the hRR acts as a transcriptional regulator of a glucocorticoid-inducible reporter gene only in the presence of retinoic acid. This induction occurs at physiological levels. Third, expression of the candidate hRR in transfected cells selectively increases the capacity of those cells to bind retinoic acid.

Development and Oncogenesis

The retinoids comprise a group of compounds including retinoic acid, retinol (vitamin A) and a series of natural and synthetic derivatives that together exert profound effects on development and differentiation in a wide variety of systems. *See* Sporn & Roberts, (1983); Mandel & Cohen, (1985); Wolback & Howe, (1925); Lotan (1980); and Fuchs & Green, (1980). Although early studies focused on the effects of retinoids on epithelial growth and differentiation, their actions have been shown to be more widespread than previously suspected. Many recent studies demonstrate the effects of these molecules on a variety of cultured cell lines including neuroblastomas (*see* Hausler, *et al.*, (1983)), melanomas (*see* Lotan, *et al.*, (1983)) and fibroblasts (*see* Shroder *et al.*, (1982)). In the human promyelocytic leukemia cells (HL-60), retinoic acid is a potent inducer of granulocyte differentiation (*see* Breitman, *et al.*, (1980)). In F9 teracarcinoma stem cells, retinoic acid will induce the differentiation of parietal endoderm, characteristic of a late mouse blastocyst (*see* Strickland & Mahdavi, (1978); Jetten *et al.*, (1979); and Wang *et al.*, (1985)). Retinoic acid has been shown to exert equally potent effects in development. For example, in the developing chick limb bud, retinoic acid is able to substitute for the action of the polarizing region in establishing the anterior-posterior axis (*see* Tickle & Eichele, (1985)). By controlling the exposure to retinoic acid, it is possible to generate novel patterns of limb structures. Although retinoic acid is primarily considered a morphogen, Northern blot analysis suggests a re-evaluation of its function in the adult. In humans, retinol deficiency has been linked to an alarming increase in a variety of cancers (*see* Moon & Itri, (1984)). Retinoids have also been shown to inhibit tumor progression in animals and block the action of tumor promoters *in vitro*. In this context, the hRR may be considered as a negative regulator of oncogenesis.

A Superfamily of Regulatory Genes

Two surprising results have emerged from the studies presented here. The first is the discovery of a family of retinoic acid receptor-related genes which predicts the existence of one or more other proteins with closely related properties (*e.g.*, the RARβ described by Brand *et al*, (1988)). Physiological studies demonstrate that both retinoic acid as well as retinol (vitamin A) can exert potent effects on cellular differentiation and that these effects are often not linked. It thus seems likely that at least one related gene product might be a specific retinol receptor or a receptor for another member of the retinoid family. The second surprising observation from these results is the close kinship of the retinoid receptor with the thyroid hormone receptor. (As we show below, the retinoic acid receptor can activate a thyroid response element or TRE; *see* the section of the specification labeled "Retinoic Acid and Thyroid Hormone Induce Gene Expression Through a Common Response Element".) This relationship is surprising in part because of the structural dissimilarity of the thyroid hormones and the retinoids. Thyroid hormones being derived from the condensation of two tyrosine molecules whereas, the retinoids are derived from mevalonic acid. The observation that chemically distinct molecules interact with receptors sharing common structures most likely reflects a common mode of action with which they elicit their particular regulatory effects. Based on this analogy, we can now propose that the interaction of retinoids with their intracellular receptors induces a cascade of regulatory events that results from the activation of specific sets of genes by the hormone/receptor complex. Although animals employ diverse means to control their development and physiology, the demonstration that the retinoic acid receptor is part of the steroid receptor superfamily suggests that mechanisms controlling morphogenesis and homeostasis may be more universal than pr viously suspected.

at position −191 of the MTV−LTR. This sequence, −169 to −200 of the rat growth hormone (rGH) gene, specifically binds thyroid hormone receptors and can confer T_3 responsiveness to a heterologous promoter (Glass *et al.* (1987)). Expression and reporter plasmids were cotransfected into CV−1 cells and *CAT* activity was measured in the absence and presence of T_3. The assays showed that neither the *alpha* nor the *beta* thyroid hormone receptor activates transcription from MTV−*CAT*, in the absence or presence of T_3. (Data not shown.) However, the addition of a TRE produces an MTV promoter that is thyroid hormone responsive. Induction of *CAT* activity is dependent on the cotransfection of a functional *alpha or beta* thyroid hormone receptor and the addition of T_3. In the presence of T_3, the *alpha* receptor (rTRα) induces *CAT* activity approximately 15−fold, while the *beta* receptor (hTRβ) induces activity by about 5−fold.

The hybrid thyroid hormone/glucocorticoid receptors were constructed to compare the functional properties of the thyroid and glucocorticoid hormone receptors. To facilitate the construction of the chimeric hybrid receptors, unique sites for the restriction enzymes *Not*I and *Xho*I were inserted flanking the DNA binding domains of hGR and hTRβ. These mutant receptors, termed hGR_{NX} and $hTR\beta_{NX}$, can be used to create hybrids with all possible combinations of amino termini, DNA−binding domains, and ligand−binding domains for these receptors. (As those skilled in the art will appreciate, comparable plasmids, such as $pRAR\alpha_{NX}$ or pMR_{NX} for example, can be used to create chimeric receptors consisting of all possible combinations of all functional domains from the various receptors in the steroid hormone receptor superfamily. The receptors and the locations of the various functional domains are shown in Figure 8.) The hybrid and parental receptors were assayed using both thyroid hormone and glucocorticoid responsive promoters, in the absence or presence of T_3 or the synthetic glucocorticoid dexamethasone.

The structures and activities of the hybrid thyroid/glucocorticoid receptors are shown in Figure 9. The receptors are divided into three sections, and hybrids are named by letters referring to the "origin" of the domain; for example, "T−G−T" has the amino and carboxyl termini of hTRβ (T−, −T) and the DNA binding domain of the hGR (−G−). Hybrids with a putative hTRβ DNA binding domain (TTG, GTT, GTG) activated transcription from TRE−*CAT*, while hybrids with an hGR DNA binding domain (GGT, TGG, TGT) activated transcription from GRE−*CAT*. This demonstrates that this region of hTRβ is analogous to the hGR DNA binding domain and is responsible for promoter recognition. Hybrid receptors with an hTRβ carboxyl terminus were activated by T_3, while those with an hGR carboxyl terminus were activated by dexamethasone. This is consistent with the identification of the carboxyl terminus as the part of the receptor that is responsible for hormone binding and activation specificity. Taken together, the functional properties of these hybrids support the assignment of the DNA−and ligand−binding domains of hTRβ.

Retinoic Acid and Thyroid Hormone Induce Gene Expression Through a Common Responsive Element

Identification of a functional retinoic acid responsive element (RARE) is crucial to our understanding of the mechanisms by which retinoic acid receptors activate gene expression and regulate cell differentiation. One impediment to such a study is the absence of any identified gene whose transcription is directly dependent on the retinoic acid receptor−hormone complex. An alternative approach to localize a RARE is to systematically challenge the inducibility of known hormonally responsive promoters with retinoic acid receptor produced from cloned cDNA. (As discussed above under the heading "The *Cis/Trans* Assay", in this system, transcriptional activation from a promoter containing a HRE is dependent on expression of functional receptor from cotransfected expression plasmids in receptorless cells such as CV−1.) Because the DNA−binding domains of the retinoic acid and thyroid hormone receptors are highly related (62% identical in their amino acid sequences, *see* Figure 6), the possibility that the retinoic acid receptor could activate gene expression through a TRE was investigated.

TRE's are known; *see*, for example, Glass, *et al*, (1987) for a discussion of a *cis*−acting element in the rat growth hormone 5' flanking genomic sequence that is necessary for thyroid hormone (3,5,3'−triiodo−L−thyronine, T_3) regulation.

To test if a TRE could effectively function as a RARE, a novel T_3 responsive promoter was constructed by replacing the glucocorticoid responsive elements present in the mouse Mammary Tumour Virus−Long Terminal Repeat (MTV−LTR) with an oligonucleotide encoding the natural TRE_{GH}. This promoter was then fused to the bacterial chloramphenicol acetyl transferase (*CAT*) gene to generate the reporter plasmid $\Delta MTV-TRE_{GH}-CAT$. After transient transfection into CV−1 cells, the inducibility of the promoter was determined by measuring *CAT* activity. When CV−1 cells are cotransfectd with the expression vector containing a human thyroid hormone receptor *beta* ($pRShT_3R\beta$) and the reporter plasmid $\Delta MTV-TRE_{GH}-CAT$, induction in *CAT* activity is observed in the presence of T_3. In contrast, cotransfection of an expression vector encoding the human glucocorticoid receptor ($pRShGR\alpha$) and the same reporter plasmid did not stimulate *CAT* activity from this promoter in response to the synthetic glucocorticoid dex−

The cells were then cultured for two days in serum free media supplemented with Nutridoma (Boehringer Mannheim) in presence or absence of inducers. CV-1 cells were then prepared for *CAT* assays as described by Gorman, *et al.* (1982) and the assays performed for 3 h using 25 μg of protein extract. All experiments with retinol were conducted in subdued light.

FIGURE 3. A, Dose-response to retinoids. CV-1 cells cotransfected with pRShRGR and pMTV*CAT* were treated with increasing concentrations of retinoids or a single 1 μM dose (*) of testosterone, dihydrotestosterone, estrogen, cortisol, aldosterone, progesterone, triiodothyronine (T_3), thyroxine (T_4), dihydroxy-vitamin D_3 (VD_3) and 25-OH-cholesterol. The levels of *CAT* activity were plotted as percentages of the maximal response observed in this experiment. B, Retinoic acid binding to cytosol extracts of transfected COS-1 cells. Bars represent bound ^{3}H-retinoic acid determined in absence (black bars) or presence (stippled bars) of a 1000-fold excess of various competitors. The values represent the mean of quadruplicate determinations. Competitors are retinoic acid (RA), retinol (R), T_4, dexamethasone (DEX) and vitamin D_3 (VD_3).

Figure 3 Methods A CV-1 cell cotransfections and *CAT* assays were performed as described in Figure 2. Retinoic acid was dissolved in a minimum volume of dimethyl sulfoxide and diluted in ethanol. All other products were diluted in ethanol and control cultures received 0.1% solvent (v/v) in media. Dose-response curves of retinoid treatment were performed in triplicate. B, Subconfluent COS-1 cells were transfected with 10 μg/dish of a control plasmid (pRS) or pRShRR by the DEAE-Dextran method (*see* Deans, *et al.*, (1984)). Cells were maintained for 2 days in DMEM with 5% charcoal-treated fetal calf serum, then harvested in TNE (40 mM tris-HC1 pH 7.5, 150 mM NaCl, 1mM EDTA) and lysed by Dounce homogenization in hypotonic buffer (50 mM tris-HCl pH 7.4, 0.1 mM EDTA, 5 mM dithiothreitol, 10 mM $NaMoO_4$, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride) and centrifuged at 100,000 X g for 30 min to yield the cytosol fraction. Incubations were performed in hypotonic buffer with 150 μg of protein from the cytosolic fraction and 2 X 10^{-8} M ^{3}H-retinoic acid (NEN, 52.5 Ci/mmole) in a total volume of 200 μl. Specific binding was measured by the addition of 2 X 10^{-5} M of competitors. Reactions were carried out at 4° C for 16 h. Bound ^{3}H-retinoic acid was quantitated using DE-81 filters. Reactions were placed on filters for 1 min and then rinsed with 5 ml of washing buffer (50 mM tris-HCl pH 7.4, 0.1 mM EDTA, 0.1 % Triton X-100). Filters were dried and counted by liquid scintillation spectrophotometry.

FIGURE 4. Southern blot analysis of human genomic DNA. A, Human placenta DNA was digested with the indicated restriction enzymes. After separation of the digested DNA in a 0.8% agarose gel (10 μg/lane) and transfer to nitrocellulose filters (*see* Southern, (1975), the blots were hybridized with an *Eco*R1 X *Pvu*II fragment from phRAR1 (~600 bp) encompassing the DNA-binding domain of the hRR under high stringency conditions (50% formamide, 5X SSPE, 1X Denhardt's, 0.1% SDS, 100 μg ml^{-1} salmon sperm DNA). The filter was washed in 0.1X SSC, 0.1% SDS at 65° C. Lambda *Hind*III DNA markers (size in Kb) are aligned to left of the autoradiogram. B, Analysis of human placenta DNA using the same probe as in A under non-stringent conditions. A parallel blot containing identical samples was hybridized as in A, except that 35% formamide was used. The filter was washed in 2XSSC, 0.1% SDS at 55° C.

FIGURE 5. Northern blot analysis of retinoic acid receptor mRNA in rat and human tissues.

Figure 5 Methods. Total RNA was isolated from various tissues using guanidine thyocyanate (*see* Chirgwin, *et al.*, (1980), separated on 1% agarose- formaldehyde gel, transferred to nitrocellulose, and hybridized under stringent conditions using the probe described in Fig. 4. Twenty μg of total RNA was used in all lanes. Migration of ribosomal RNA's (28S and 18S) are indicated for size markers. The nitrocellulose filter was autoradiographed at -70° C with an intensifying screen for 1 week.

FIGURE 6. Schematic amino acid comparisons of the hGR, hRR and $hT_3R\beta$ structures. Amino acid sequences have been aligned schematically with the percentage amino acid identity for each region of homology in the intervals between dotted lines.

FIGURE 7 is a schematic diagram of a generalized steroid\thyroid\retinoic acid receptor gene, showing the division of the gene into regions A/B, C, D, and E. The function of the A/B region is just beginning to be elucidated; the C region encodes the DNA-binding domain; the D region is believed to be a hinge region; and the E region encodes the ligand-binding domain.

FIGURE 8 is a schematic drawing that shows amino acid comparison of members of the steroid hormone receptor superfamily. Primary amino acid sequences have been aligned on the basis of regions of maximum amino acid similarity, with the percentage amino acid identity indicated for each region in relation to the hGR (Miller *et al.*, (1985). Domains shown are: a domain at the NH_2-terminal nd that is required for "maximum activity"; the 66- to 68-amino acid DNA-binding domain core ("DNA"); and the 250-amino acid ligand-binding (or hormone-binding domain) ("Hormone"). The amino acid position of each domain boundary is shown. Amino acid numbers for all receptors represent the human forms with the exception of v-*erb*-A and E75 (Segraves, 1988). Functional assignments have been determined by characterization of

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SPECIFICATION SUMMARY

From the foregoing description, one of ordinary skill in the art can understand that the present invention provides substantially pure DNA which encodes the retinoid receptor protein referred to as retinoic acid receptor protein. The invention also provides a plasmid containing retinoic acid receptor DNA. This plasmid, phRAR1, has been deposited with the American Type culture Collection for patent purposes.

The invention is also comprised of retinoic acid receptor proteins, including modified functional forms thereof, expressed from the DNA (or mRNA) of the invention.

7. A method according to Claim 4 (a) (2) wherein the reporter gene is selected from the group consisting of a chloramphenicol acetyltransferase (CAT) gene and a firefly luciferase gene.

8. A method according to Claim 4 (a) (2) wherein the hormone response element is selected from the group consisting of wild-type, (1) glucocorticoid response element, (2) thyroid response element, (3) mineralocorticoid response element, (4) estrogen-related response element, (5) retinoic acid response element, and (6) vitamin D_3 response element.

9. A method according to Claim 8 wherein the glucocorticoid response element is part of the mammary tumor virus long terminal repeat sequence (MTV LTR), and the thyroid response element is part of the growth hormone promoter sequence.

10. A method according to Claim 4 wherein the ligand(s) identified in step (d) are corroborated by evaluating the binding properties of the chimeric receptor and the ligand(s) identified in step (d).

FIG. 1B-2

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                91                  101                 111
    ProCysPheValCysGlnAspLysSerSerGlyTyrHisTyrGlyValSerAlaCysGluGlyCysLysGlyPhePheArgArgSerIle
361 CCTTGCTTTGTCTGTCAGGACAAGTCCTCAGGCTACCACTATGGGGTCAGCGCCTGTGAGGGCTGCAAGGGCTTCTTCCGCCGCAGCATC

               121                 131                 141
    GlnLysAsnMetValTyrThrCysHisArgAspLysAsnCysIleIleAsnLysValThrArgAsnArgCysGlnTyrCysArgLeuGln
451 CAGAAGAACATGGTGTACACGTGTCACCGGGACAAGAACTGCATCATCAACAAGGTGACCCGGAACCGCTGCCAGTACTGCCGACTGCAG

               151                 161                 171
    LysCysPheGluValGlyMetSerLysGluSerValArgAsnAspArgAsnLysLysLysLysGluValProLysProGluCysSerGlu
541 AAGTGCTTTGAAGTGGGCATGTCCAAGGAGTCTGTGAGAAACGACCGAAACAAGAAGAAGAAGGAGGTGCCCAAGCCCGAGTGCTCTGAG

               181                 191                 201
    SerTyrThrLeuThrProGluValGlyGluLeuIleGluLysValArgLysAlaHisGlnGluThrPheProAlaLeuCysGlnLeuGly
631 AGCTACACGCTGACGCCGGAGGTGGGGGAGCTCATTGAGAAGGTGCGCAAAGCGCACCAGGAAACCTTCCCTGCCCTCTGCCAGCTGGGC

               211                 221                 231
    LysTyrThrThrAsnAsnSerSerGluGlnArgValSerLeuAspIleAspLeuTrpAspLysPheSerGluLeuSerThrLysCysIle
721 AAATACACTACGAACAACAGCTCAGAACAACGTGTCTCTCTGGACATTGACCTCTGGGACAAGTTCAGTGAACTCTCCACCAAGTGCATC

               241                 251                 261
    IleLysThrValGluPheAlaLysGlnLeuProGlyPheThrThrLeuThrIleAlaAspGlnIleThrLeuLeuLysAlaAlaCysLeu
811 ATTAAGACTGTGGAGTTCGCCAAGCAGCTGCCCGGCTTCACCACCCTCACCATCGCCGACCAGATCACCCTCCTCAAGGCTGCCTGCCTG

               271                 281                 291
    AspIleLeuIleLeuArgIleCysThrArgTyrThrProGluGlnAspThrMetThrPheSerAspGlyLeuThrLeuAsnArgThrGln
901 GACATCCTGATCCTGCGGATCTGCACGCGGTACACGCCCGAGCAGGACACCATGACCTTCTCGGACGGGCTGACCCTGAACCGGACCCAG

               301                 311                 321
    MetHisAsnAlaGlyPheGlyProLeuThrAspLeuValPheAlaPheAlaAsnGlnLeuLeuProLeuGluMetAspAspAlaGluThr
991 ATGCACAACGCTGGCTTCGGCCCCCTCACCGACCTGGTCTTTGCCTTCGCCAACCAGCTGCTGCCCCTGGAGATGGATGATGCGGAGACG

               331                 341                 351
    GlyLeuLeuSerAlaIleCysLeuIleCysGlyAspArgGlnAspLeuGluGlnProAspArgValAspMetLeuGlnGluProLeuLeu
1081 GGGCTGCTCAGCGCCATCTGCCTCATCTGCGGAGACCGCCAGGACCTGGAGCAGCCGGACCGGGTGGACATGCTGCAGGAGCCGCTGCTG
```

FIG. 2A

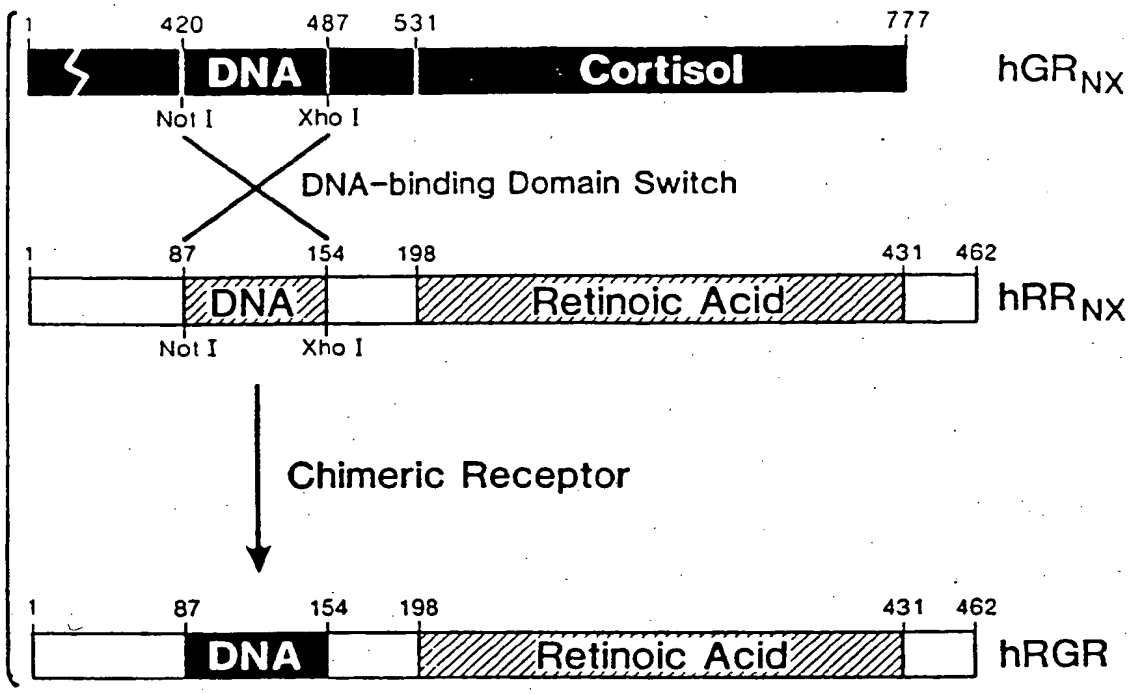
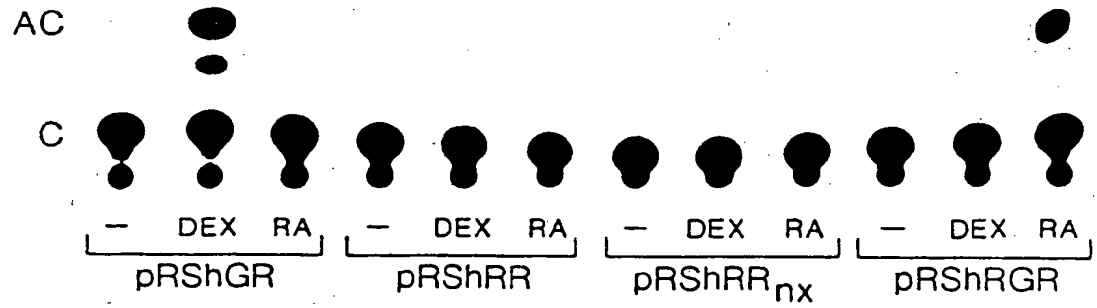


FIG. 2B



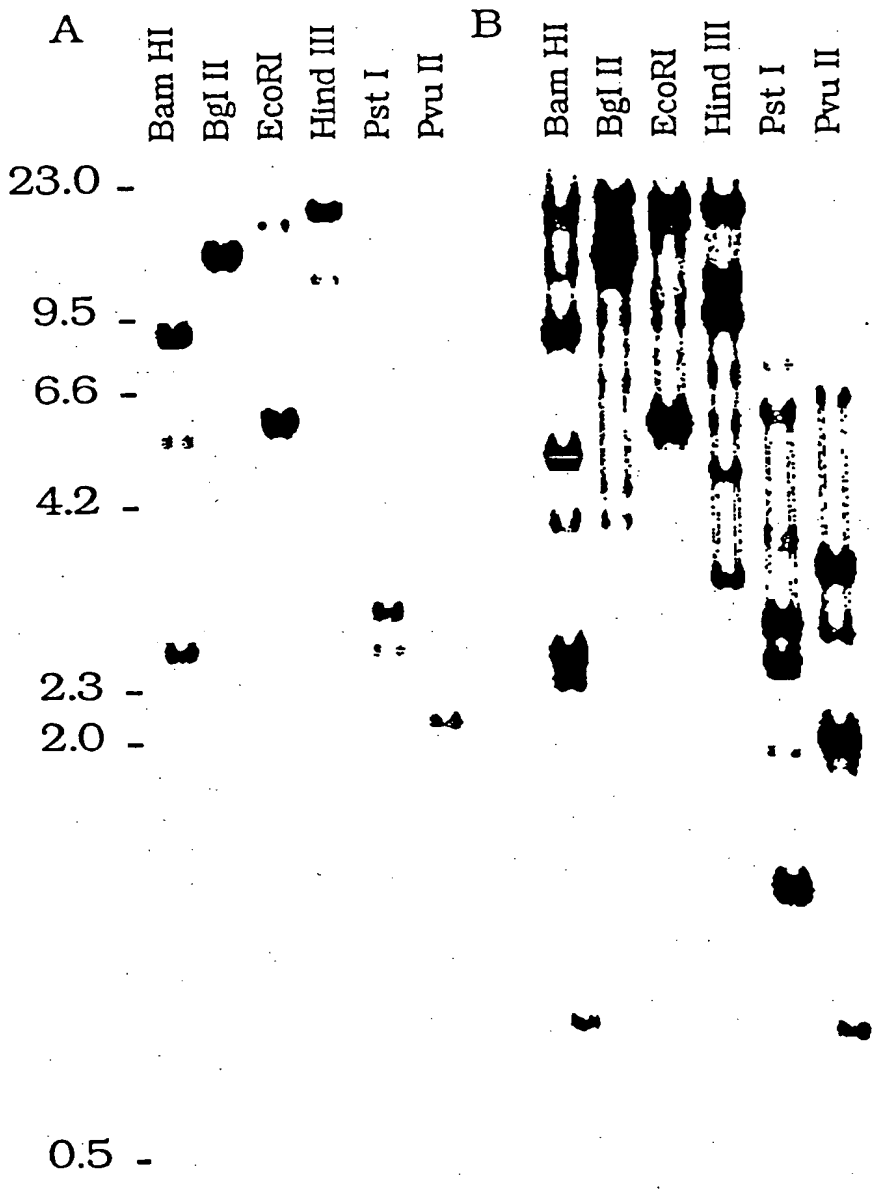


FIGURE 4

FIG. 6

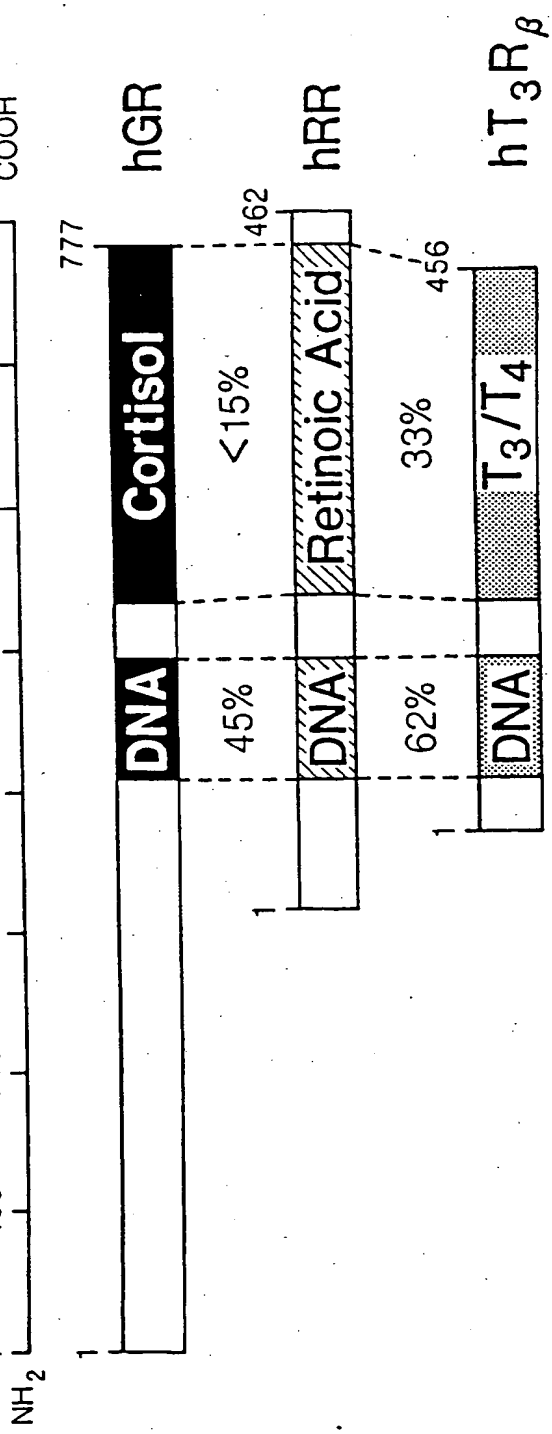
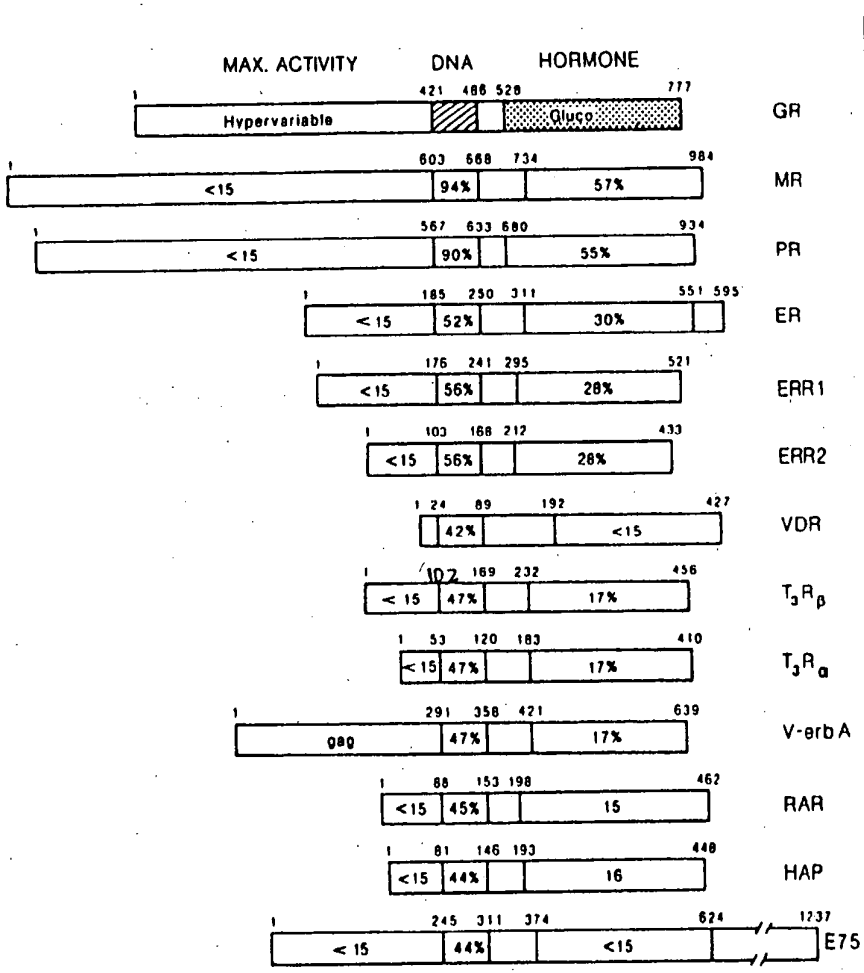


FIGURE 8.



	HRE	DNA BINDING	HORMONE BINDING IN VITRO	HORMONE BINDING IN VIVO	TRANS-ACTIVATION	CHROMO-SOME	SPECIES
GR	+[15-19]	+[16,17,21]	+[26,82]	+[48,57, 77]	+[48,57, 78,79]	5[26]	h,[26] r,[77] m.[78]
MR	nd	nd	nd	+[36]	+[36]	4[36]	h[36]
PR	+[24,34]	+[24,34]	nd	nd	+[34]	11[79]	rabbit,[32] h,[33] c,[34]
ER	+[22,23]	+[23,62]	nd	+[23,53, 62]	+[53,62]	6[62]	h,[29] c,[30] frog[31]
ERR1	nd	nd	nd	nd	nd	nd	h[39]
ERR2	nd	nd	nd	nd	nd	nd	h[39]
VDR	nd	nd	nd	+[35]	nd	nd	h,[35] c[35]
T_3R_β	+[25]	+[25]	+[37]	nd	+[60]	3[37]	h[37]
T_3R_α	nd	nd	+[38,40]	nd	+[60]	17[40]	r,[40] h,[41] c[38]
V-erbA	+	+	(−)[38]	nd	nd	virus	c[28]
RAR	nd	nd	nd	+[42,43]	+[42,43]	17[83]	h[42,43]
HAP	nd	nd	nd	nd	nd	3[45]	h[45]
E75	nd	nd	nd	nd	nd		d[46]

EP 0 540 065 A1

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 92 12 1951

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
D,X	NATURE vol. 325, 1 January 1987, LONDON GB pages 75 - 78 GREEN, S. & CHAMBON, P. 'Oestradiol induction of a glucocorticoid-responsive gene by a chimaeric receptor' * the whole document *	1,3 1-10	C12N15/12 C12N15/62 G01N33/68 G01N33/74 C12N5/10
Y	---		
P,D,X Y	WO-A-8 803 168 (THE SALK INSTITUTE FOR BIOLOGICAL STUDIES) * the whole document *	1,3-10 1-10	

T	NATURE vol. 330, 3 December 1987, LONDON GB pages 420 - 421 ROBERTSON, M. 'Towards a biochemistry of morphogenesis' * the whole document *	1-10	

P,X,D	NATURE vol. 330, 17 December 1987, LONDON GB pages 624 - 629 GIGUERE, V. ET AL. 'Identification of a receptor for the morphogen retinoic acid' * the whole document *	4-10	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C12N G01N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	01 MARCH 1993	ANDRES S.M.

CATEGORY OF CITED DOCUMENTS

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